SUBSTITUTE FORM PTO-1390 U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
10848-018001

U S. APPLICATION NO. (If Known, see 37 CFR 1.5) 10/049574

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE00/02748	11 August 2000	14 August 1999

TITLE OF INVENTION
METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING STATUS

APPLICANT(S) FOR DO/EO/US
Wolf Bertling

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☒ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☒ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))

Items 11 to 16 below concern other documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1 98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    ☒ PCT International Search Report (7 pages) with seven references attached
    ☐
    ☐
    ☐
    ☐

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14 February 2002
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VINCE DEFANTE
Typed Name of Person Signing

U.S. APPLICATION NO. (IF KNOWN) 10/049574	INTERNATIONAL APPLICATION NO. PCT/DE00/02748	ATTORNEY'S DOCKET NUMBER 10848-018001

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)- (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100**

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Claims	Number Filed	Number Extra	Rate		
Total Claims	16 - 20 =		x **$18**	$0.00	
Independent Claims	1 - 3 =		x **$84**	$0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ **$280**	$0.00	
			TOTAL OF ABOVE CALCULATIONS =	$0.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2				$445.00	
			SUBTOTAL =	$445.00	
Processing fee of **$130** for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))				$0.00	
			TOTAL NATIONAL FEE =	$445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$0.00	
			TOTAL FEES ENCLOSED =	$445.00	
			Amount to be refunded:	$	
			Charged:	$	

a. ☒ A check in the amount of $445.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 06-1050 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

Attorney's Docket No.: 10848-018001 / 412023GA-go

#3/a

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Wolf Bertling
Serial No. : To Be Assigned
Filed : 14 February 2002
Title : METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING STATUS

Art Unit : Unknown
Examiner : Unknown

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Specification:

Please add the following paragraph to the application after the title:

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a National Stage application under 35 U.S.C. §371 and claims benefit under 35 U.S.C. §119(a) of International Application No. PCT/DE00/02748 having an International Filing Date of August 11, 2000, which claims benefit of DE 199 37 654.9 filed on August 14, 1999 and DE 199 41 447.5 filed on August 31, 1999.--

Please delete the paragraph on page 3, lines 29-31.

CERTIFICATE OF MAILING BY EXPRESS MAIL

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February 14, 2002
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Vince Defante
Typed or Printed Name of Person Signing Certificate

In the Claims:

Please amend the claims as follows. A full set of pending claims are shown herein for convenience.

1. (Amended) A method for indirectly determining blood clotting status having the following steps:

a) providing a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent γ-carboxylase,

b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),

c) forming a first ratio (R1) from the first (C1) and second concentration (C2) or forming a second ratio (R2) from the third (C3) and first concentration (C1) or forming a third ratio (R3) from the third (C3) and second concentration (C2),

where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

$$C3 - C2 = C1$$

and

d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

2. The method as claimed in claim 1, where in step b) additionally at least a first competitor (K1) is used to measure the first concentration (C1), a second competitor (K2) is used to measure the second concentration (C2) or a third competitor (K3) is used to measure the third concentration (C3).

3. (Amended) The method as claimed in claim 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a labeling substance.

4. (Amended) The method as claimed in claim 2, where in place of measuring the at least two concentrations as in step b), a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

5. The method as claimed in claim 4, where the combined signal is a combined color generated in particular by fluorescent dyes, a fluorescent signal elicited by the Förster effect or a reduction caused by the quencher in a fluorescent signal.

6. (Amended) The method as claimed in claim 1, where the body fluid is plasma, blood, saliva, urine or the like.

7. (Amended) The method as claimed in claim 1, where the measurement of the first (C1), second (C2) and/or third concentration (C3) or of the combined signal takes place by an immunological method.

8. (Amended) The method as claimed in claim 7, where in the immunological method, at least one of the antibodies (A1, A2, A3) is immobilized on a support.

9. (Amended) The method as claimed in claim 1, where the first (C1), second (C2) and/or third concentration (C3) and/or the combined signal is measured by means of a color reaction or fluorescence detection.

10. (Amended) The method as claimed in claim 1, where the protein which can be modified by a vitamin K-dependent γ-carboxylase is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

11. (Amended) A kit for carrying out the method as claimed in claim 1, having a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein and having a second antibody (A2) for immunological determination of a second concentration (C2) of the decarboxylated form of the protein, characterized in that the first (A1) and second antibodies (A2) is in each case conjugated to a labeling substance, where the labeling substances are selected so that they are able together to generate a combined signal.

12. A kit as claimed in claim 11, where the labeling substance is an enzyme, a fluorescent dye or a quencher.

13. (Amended) A kit as claimed in claim 11, where the combined signal is a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal.

14. (Amended) A kit as claimed in claim 11, where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

Please cancel claims 15-23 without prejudice to continued prosecution.

Please add the following new claims:

24. The method of claim 3, wherein the labeling substance is selected from the group consisting of an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin, and avidin.

25. The method of claim 8, wherein the support is selected from the group consisting of a plastic, a magnetic particle, a latex particle, a gold particle, a test strip, and a membrane.

In the Abstract:

Please add the attached Abstract to the application after the claims.

REMARKS

Applicant respectfully requests entry of the amendments and remarks submitted herein. Claims 1, 3-4, 6-11, and 13-14 have been amended, claims 15-23 have been canceled, and new claims 24-25 have been added. Support for the amendments and for new claims 24-25 can be found in the originally filed claims and throughout the specification. Therefore, claims 1-14 and 24-25 are currently pending. Attached is a marked-up version of the changes being made by the current amendments. Reconsideration of the pending application is respectfully requested.

In addition, Applicants amended the specification to include a paragraph describing related applications and to claim the benefit of priority to such applications. Applicants also have amended the specification to remove the paragraph on page 3 that refers to claim numbers, and to add an Abstract. The attached Abstract is the English language Abstract that was published with the PCT application. Therefore, Applicants submit that there is no new matter introduced by these amendments.

CONCLUSION

Applicant asks that claims 1-14 and 24-25 be examined. The enclosed filing fee takes into account the new claims added by this Preliminary Amendment. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: February 14, 2002

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

The paragraph on page 3, lines 29-31 has been deleted.

In the Claims:

Claims 1, 3-4, 6-11, and 13-14 have been amended as follows:

1. (Amended) A method for indirectly determining [the] blood clotting status having the following steps:

a) providing [provision of] a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent γ-carboxylase,

b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),

c) forming a first ratio (R1) from the first (C1) and second concentration (C2) or forming a second ratio (R2) from the third (C3) and first concentration (C1) or forming a third ratio (R3) from the third (C3) and second concentration (C2),

where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

$$C3 - C2 = C1$$

and

d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

3. (Amended) The method as claimed in claim [1 or] 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a

labeling substance[, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, a biotin, streptavidin or avidin].

4. (Amended) The method as claimed in claim 2 [any of the preceding claims], where in place of measuring the at least two concentrations as in step b), a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

6. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the body fluid is plasma, blood, saliva, urine or the like.

7. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the measurement of the first (C1), second (C2) and/or third concentration (C3) or of the combined signal takes place by an immunological method.

8. (Amended) The method as claimed in claim 7, where in the immunological method, at least one of the antibodies (A1, A2, A3) is immobilized on a support[, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane].

9. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the first (C1), second (C2) and/or third concentration (C3) and/or the combined signal is measured by means of a color reaction or fluorescence detection.

10. (Amended) The method as claimed in claim 1 [any of the preceding claims], where the protein which can be modified by a vitamin K-dependent γ-carboxylase is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

11. (Amended) A kit for carrying out the method as claimed in claim 1 [any of the preceding claims], having a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein and having a second antibody (A2) for immunological determination of a second concentration (C2) of the decarboxylated form of the protein, characterized in that the first (A1) and second antibodies (A2) is in each case conjugated to a labeling substance, where the labeling substances are selected so that they are able together to generate a combined signal.

13. (Amended) A kit as claimed in claim 11 [or 12], where the combined signal is a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal.

14. (Amended) A kit as claimed in [any of claims] claim 11 [to 13], where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

Claims 15-23 have been canceled.

New claims 24-25 have been added.

In the Abstract:

The Abstract on the attached page has been added to the application.

ABSTRACT OF THE DISCLOSURE

The invention relates to a method for indirectly determining the blood-clotting status. The inventive method comprises the following steps: a) collecting body fluids which contain a protein that can be modified by a vitamin K-dependent γ-carboxylase, b) determining at least two concentrations selected from a group consisting of a first concentration C1 of carboxylated protein, a second concentration C3 of decarboxylated protein and an entire concentration C3 of carboxylated and decarboxylated protein, whereby the first concentration C1 is determined using a first antibody A1, the second concentration using a second antibody A2 and the third concentration C3 using a third antibody A3, c) generating a first quotient Q1 from the first C1 and second C2 concentration or generating a second quotient Q2 from the third C3 and first C1 concentration or generating a third quotient Q3 from the third C3 and second concentration C2, whereby a concentration C1, C2, C3 which has not been determined in step b) and which is required for generating the first Q1, the second Q2, or the third quotient Q3 is calculated according to the following relation: C3-C2=C1 and d) the first, second or third quotient Q1, Q2, Q3 are correlated with the blood-clotting status.

Method for indirectly determining the blood clotting status

The invention relates to a method for indirectly determining the blood clotting status.

The blood clotting status can be determined indirectly by measuring the prothrombin concentration in human body fluids. Prothrombin is a protein which occurs chiefly in human blood plasma. This protein can be modified by a vitamin K-dependent γ-carboxylase. Prothrombin is partly responsible for blood clotting. It converts fibrinogen into fibrin.

The fibrinogen conversion induced by prothrombin takes place only when prothrombin is present in natural carboxylated form. The carboxylation is effected in the liver by a carboxylase with binding of the cofactor vitamin K. The carboxylase activity depends on the vitamin K concentration. If the carboxylase activity is reduced, an abnormal, noncarboxylated form of prothrombin which has no clotting activity is produced.

In healthy people, prothrombin is present in the natural, i.e. carboxylated, form. Vitamin K acts as cofactor in the carboxylation. In people who are ill, especially in people with liver damage, or on addition of anticoagulants, prothrombin also occurs in the abnormal form.

Carboxylated prothrombin brings about clotting only if Ca^{2+} ions are bound beforehand. Only then is the carboxylated prothrombin able to bind to the membranes of blood platelets and bring about clotting. Only the carboxylated form of prothrombin is able to bind calcium. The blood clotting status can thus be inferred from the content of carboxylated prothrombin.

US 4,769,320 discloses a method in which the content of carboxylated prothrombin is measured by use of antibodies in an immunoassay. The antibodies are specific for carboxylated prothrombin in the presence of calcium. They do not bind to decarboxylated prothrombin. A kit for determining the level of carboxylated prothrombin in a plasma sample is described and contains such an antibody.

US 5,252,712 discloses a monoclonal antibody which is specific for noncarboxylated prothrombin. It is possible by use of this antibody in an immunoassay to measure the concentration of uncarboxylated prothrombin. This also makes it possible to gain information about the blood clotting status.

US 4,780,410 discloses a sandwich immunoassay method for quantifying decarboxylated prothrombin. An immobilized monoclonal antibody directed against decarboxylated prothrombin is used in the method. Decarboxylated prothrombin binding thereto is detected by a second antibody directed against prothrombin. A kit for carrying out the method is also described.

Kornberg A. et al., Circulation 88 (1993), pages 454 - 460, disclose determination of the concentration of carboxylated prothrombin in a sample by use of a competitor. In this case, peroxidase-labeled prothrombin competes as competitor with the prothrombin in the sample for binding to an immobilized anti-prothrombin antibody. The peroxidase-labeled prothrombin bound to the anti-prothrombin antibody can be detected by an enzyme reaction. The size of the signal produced thereby is inversely proportional to the prothrombin concentration in the sample.

JP 05 284 994 A discloses three monoclonal antibodies. A first one binds specifically to human decarboxylated prothrombin, a second one binds specifically to human

prothrombin, human thrombin and human decarboxylated prothrombin and a third one binds specifically to human decarboxylated prothrombin and human prothrombin.

The determination of decarboxylated prothrombin in blood by means of an ELISA using a monoclonal antibody is disclosed by von Kries, R. et al., Thrombosis and Haemostatis 68 (1992), pages 383 - 387.

The problem which arises in the prior art is that the sample material to be analyzed is not always analyzed immediately after the sample is taken. 1 to 2 days elapse on occasion through transport of the sample material. Most of the factors involved in blood clotting are highly sensitive and rapidly inactive. During this time, inter alia both carboxylated and noncarboxylated prothrombin in the sample is broken down. The consequence is falsification of the results of the blood clotting status.

It is an object of the invention to eliminate the disadvantages of the prior art. It is intended in particular to increase the accuracy of measuring the blood clotting status by determination of the prothrombin content. It is further intended to provide a kit which makes possible more accurate measurement of the blood clotting status.

This object is achieved by the features of claims 1 and 11. Expedient developments of the invention are evident from the features of claims 2 to 10 and 12 to 20.

According to the invention, a method for indirectly determining the blood clotting status having the following steps is provided:

a) removal of body fluid which contains a protein which can be modified by a vitamin K-dependent γ-carboxylase,

b) measuring at least two concentrations selected from a group consisting of a first concentration of carboxylated protein, a second concentration of decarboxylated protein and a total concentration of carboxylated and decarboxylated protein, where the first concentration is measured using a first antibody, the second concentration is measured using a second antibody and the third concentration is measured using a third antibody,

c) forming a first ratio from the first and second concentration

or

forming a second ratio from the third and first concentration

or

forming a third ratio from the third and second concentration,

where a concentration which is necessary for forming the first, second or third ratio and is not measured in step b) is calculated in accordance with the following relation:

$$C3 - C2 = C1$$

and

d) correlating the first, second or third ratio with the blood clotting status.

A protein which can be modified by a vitamin K-dependent γ-carboxylase means a protein which, depending on the blood clotting status, may be present in proportions

both of the carboxylated and of the decarboxylated form. The protein may be a protein which can easily be obtained from a patient, e.g. a protein from the saliva. The protein may be a protein which is subject to the same percentage hypomodification as prothrombin. An antibody may be, for the purpose of the invention, an antibody, an antibody fragment or another substance with binding specificity for the carboxylated form, the decarboxylated form or both forms of the protein.

It is possible with the method of the invention to measure accurately the blood clotting status on the basis of the content of modifiable protein. Errors in the determination of the blood clotting status are minimized through taking account both of the content of carboxylated protein and of the content of decarboxylated protein and the relating of the two aforementioned protein contents.

In one embodiment of the invention, in step b) additionally at least a first competitor is used to measure the first concentration, a second competitor is used to measure the second concentration or a third competitor is used to measure the third concentration. The competitor is a substance which competes with the carboxylated protein, the decarboxylated protein or the carboxylated and decarboxylated protein for binding to one of the antibodies. The competitor may be the carboxylated or decarboxylated protein, in which case it is provided with a labeling substance. In place of the complete protein it is also possible to use a fragment of this protein as competitor.

It is preferred for at least one of the antibodies or at least one of the competitors to be conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin. Any enzyme detectable by means of an enzymatic reaction can be

used as enzyme. The labeling of the antibody with a gold particle permits the bound antibody to be detected by a plasmon resonance method.

In place of measuring the at least two concentrations as in step b) it is also possible for a combined signal correlating therewith to be generated and measured by using two antibodies selected from a group consisting of the first, the second and the third antibody and, where appropriate, at least one of the competitors, and be directly correlated with the blood clotting status. The combined signal is produced by different individual signals acting together. It corresponds to the first, second or third ratio. The formation of these ratios as in step c) is dispensed with. This makes it possible to carry out the method more quickly and simply. The combined signal may be a combined color, generated in particular by fluorescent dyes, a fluorescent signal elicited by the Förster effect or a reduction caused by the quencher in a fluorescent signal. The combined color may also be generated by two enzymes which each catalyze a specific color reaction. The Förster effect involves a radiationless energy transfer from an excited first fluorophore to a directly adjacent second fluorophore. The first fluorophore thus makes the transition to the ground state, while the second fluorophore is excited and fluoresces. In the method of the invention it is possible, for example, for the first and second antibodies to be conjugated with fluorophores which make the first effect possible. The binding of the first antibodies directly adjacent to the second antibodies can be established by means of a fluorescent signal elicited by the Förster effect. When there is a radiationless energy transfer from fluorophore to quencher the fluorescence is quenched. The total measurable fluorescent signal is thus reduced.

The body fluid may expediently be plasma, blood, saliva, urine or the like. All body fluids in which the modifiable protein is present in a content which makes measurement possible are suitable in principle.

According to one developmental feature of the invention, the measurement of the first, second and/or third concentration or of the combined signal takes place by an immunological method. The immunological method in this case may involve at least one of the antibodies being immobilized on a support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane. The plastic may be in the form of a test tube, a test strip, a plastic particle or a well of a microtiter plate.

The first, second and/or third concentration and/or the combined signal can be measured by means of a color reaction or fluorescence detection. This makes possible particularly rapid and simple measurement of the blood clotting status.

The protein which can be modified by a vitamin K-dependent γ-carboxylase is preferably one of the clotting factors which are referred to in the decarboxylated form as "proteins induced by vitamin K antagonism or absence" (PIVKA factors): prothrombin, factor VII, factor IX or factor X, or is nephrocalcin or osteocalcin. It is also possible to use for determining the blood clotting status other proteins which are carboxylated by a vitamin K-dependent carboxylase and can likewise be influenced by anticoagulants which can be administered orally. Nephrocalcin is, for example, detectable in urine. It is unnecessary to take any blood on use of this protein. This means a considerable alleviation for patients whose blood clotting status must be monitored continously.

Also provided is a kit for carrying out the method of the invention, comprising at least two antibodies selected from a group consisting of a first antibody for immunological determination of a first concentration of the carboxylated form of the protein, a second antibody for immunological determination of a second concentration of the decarboxylated form of the protein and a third antibody for immunological determination of a total concentration of carboxylated and decarboxylated protein.

The first, second and third antibody may be antibodies known in the prior art. Such antibodies are disclosed, for example, in US 5,252,712 and US 4,769,320, the contents of which are incorporated herein by reference.

The kit may additionally comprise at least a first competitor for measuring the first concentration, a second competitor for measuring the second concentration or a third competitor for measuring the third concentration. At least one of the antibodies or competitors present in the kit may be conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin.

The first and the second antibody are preferably immobilized on a support. The support may be a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane. If the support is a test strip, the first and the second antibody may each be absorbed on a separate field on the test strip. The kit preferably comprises a third antibody conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin. This makes it possible to measure the respective content particularly simply, for example by means of a color reaction on the test strip.

In a further development of the invention, the third antibody is immobilized on the or another support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane. If the support is the or another test strip, the third antibody can be absorbed on a field of the test strip. The kit preferably comprises a first and second antibody in each case conjugated to a labeling substance, in particular an enzyme, a fluorescent dye or a quencher, where the labeling substances are selected so that they are able together to generate a combined signal, in particular a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal. The combined signal corresponds to the first ratio.

The protein is preferably prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

The method of the invention is explained hereinafter by means of the drawing:

Fig. 1 shows the correlation of the blood clotting status with cPT/dcPT,

Fig. 2 shows the correlation of the blood clotting status with dcPT/cPT and

Fig. 3 shows the correlation of the blood clotting status with dcPT.

Fig. 1 is a plot of the ratio of the concentrations of carboxylated and decarboxylated prothrombin against the blood clotting status INR. The concentrations in this case are measured as OD values. The blood clotting status INR which results from a measured ratio of 0.5 is 3.8.

Fig. 2 is a plot of the ratio of the concentrations of decarboxylated and carboxylated prothrombin against the blood clotting status. It is evident that the ratio in this case correlates particularly well with the blood clotting status INR.

Fig. 3 shows the correlation known from the prior art of dcPT with the blood clotting status INR. This changes with increasing age of the samples.

Example 1:
The so-called ELISA on a microtiter plate is particularly suitable for serial measurements.

a) Sample preparation:
The cavities of a microtiter plate (Maxisorb, NUNC) are each coated with 50 µl of an antibody (10 µg/ml in carbonate buffer) at 4°C overnight. The cavities are washed three times with PBS. Nonspecific binding sites are saturated with 50 µl of 1% BSA in PBS per well at room temperature for 1 hour. The cavities are then washed three times with PBS/0.05% Tween 20.

The following samples are then loaded, each diluted 1:50 in PBS/0.1% BSA (50 µl/cavity):

calibration plasmas,
normal plasma,
patient's plasma and
prothrombin-deficient plasma (negative control).

The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20. 50 µl/cavity of rabbit anti-complete prothrombin (10 µg/ml) are added. The mirotiter plate is then incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20. 50 µl/cavity of goat anti-rabbit antibody, biotin-conjugated (Dionova, 1:20 000

in PBS/0.1% BSA) are added. The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20.

50 µl/cavity of strepvatidin-peroxidase conjugate (Roche Diagnostics, 1:1000 in conjugate buffer) are added. The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20.

To carry out the development reaction, 50 µl/cavity of ABTS solution (Roche Diagnostics, 1 mg/ml) are added. The microtiter plate is incubated at room temperature for half an hour to one hour. The absorptions (OD values) are measured in an ELISA reader.

It is self-evident that the method can be considerably shortened if the anti-prothrombin antibody used for detecting bound prothrombin already has a labeling substance such as peroxidase or another enzyme. A further shortening of the method can be achieved by using a directly detectable labeling substance such as a fluorophore. No development reaction is necessary on use of such a labeling substance.

b) Evaluation:

The following are determined:

aa) the OD values of the complete prothrombin (cavities are coated with monoclonal anti-complete prothrombin antibodies),

bb) the OD values of the decarboxylated prothrombin (cavities are coated with monoclonal anti-decarboxy-prothrombin antibodies) and

cc) the OD values of the carboxylated prothrombin (difference between the OD values of the complete

prothrombin and the OD values of the decarboxylated prothrombin).

Then calibration plots are constructed from the measured OD values (see figs. 1 - 3: points A, B, C and D) of the calibration plasmas, and the INR of the patient's plasmas calculated.

It is self-evident that the clotting status can also be measured by measuring the corresponding concentrations of proteins other than prothrombin which can be modified by a vitamin K-dependent γ-carboxylase. Examples of such proteins are factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

Example 2:

The cavities of a microtiter plate (Maxisorb, NUNC) are coated with in each case 50 µl of an antibody directed against carboxylated and decarboxylated prothrombin or of an antibody directed only against decarboxylated prothrombin (10 µg/ml in carbonate buffer) at 4°C overnight. The cavities are washed three times with PBS. Nonspecific binding sites are saturated with 50 µl of 1% BSA in PBS per cavity at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20.

Peroxidase-labeled decarboxylated prothrombin is pulled together with the following samples, each diluted 1:50 in PBS/0.1% BSA, in a final concentration of 30 µg/ml into the antibody-coated cavities of the microtiter plate (50 µl/cavity):

calibration plasmas,
normal plasma,
patient's plasma and
prothrombin-deficient plasma (negative control).

The microtiter plate is incubated at room temperature for one hour. The cavities are then washed three times with PBS/0.05% Tween 20. 50 µl/cavity of ABTS solution (Roche Diagnostics, 1 mg/ml) are added. The microtiter plate is incubated at room temperature for half an hour to one hour. The OD values in the cavities of the microtiter plate are measured in an ELISA reader. A higher OD value in a cavity means a lower concentration of prothrombin in the particular sample. The prothrombin concentration in the patient's plasma is measured by means of a calibration plot constructed using the calibration plasmas. Cavities coated with the antibody against carboxylated and decarboxylated prothrombin are used to determine the total concentration of carboxylated and decarboxylated prothrombin. Cavities coated with the antibody directed against decarboxylated prothrombin are used to determine the concentration of decarboxylated prothrombin. The ratio of the measured total concentrations of carboxylated and decarboxylated and the concentration of decarboxylated prothrombin is found. The clotting status can be measured from the resulting ratio on the basis of the ratios for the calibration plasmas.

New claims

1. A method for indirectly determining the blood clotting status having the following steps:

a) provision of a sample of body fluid which contains a protein which can be modified by a vitamin K-dependent γ-carboxylase,

b) measuring at least two concentrations selected from a group consisting of a first concentration (C1) of carboxylated protein, a second concentration (C2) of decarboxylated protein and a total concentration (C3) of carboxylated and decarboxylated protein, where the first concentration (C1) is measured using a first antibody (A1), the second concentration is measured using a second antibody (A2) and the third concentration (C3) is measured using a third antibody (A3),

c) forming a first ratio (R1) from the first and second concentration (C2)

or

forming a second ratio (R2) from the third (C3) and first concentration (C1)

or

forming a third ratio (R3) from the third (C3) and second concentration (C2),

where a concentration (C1, C2, C3) which is necessary for forming the first (R1), second (R2) or third (R3) ratio and is not measured in step b) is calculated in accordance with the following relation:

C3 - C2 = C1

and

d) correlating the first, second or third ratio (R1, R2, R3) with the blood clotting status.

2. The method as claimed in claim 1, where in step b) additionally at least a first competitor (K1) is used to measure the first concentration (C1), a second competitor (K2) is used to measure the second concentration (C2) or a third competitor (K3) is used to measure the third concentration (C3).

3. The method as claimed in claim 1 or 2, where at least one of the antibodies (A1, A2, A3) or at least one of the competitors (K1, K2, K3) is conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, a biotin, streptavidin or avidin.

4. The method as claimed in any of the preceding claims, where in place of measuring the at least two concentrations as in step b) a combined signal correlating therewith is generated and measured by using two antibodies selected from a group consisting of the first (A1), the second (A2) and the third antibody (A3) and, where appropriate, at least one of the competitors (K1, K2, K3), and is directly correlated with the blood clotting status.

5. The method as claimed in claim 4, where the combined signal is a combined color generated in particular by fluorescent dyes, a fluorescent

signal elicited by the Förster effect or a reduction caused by the quencher in a fluorescent signal.

6. The method as claimed in any of the preceding claims, where the body fluid is plasma, blood, saliva, urine or the like.

7. The method as claimed in any of the preceding claims, where the measurement of the first (C1), second (C2) and/or third concentration (C3) or of the combined signal takes place by an immunological method.

8. The method as claimed in claim 7, where in the immunological method at least one of the antibodies (A1, A2, A3) is immobilized on a support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane.

9. The method as claimed in any of the preceding claims, where the first (C1), second (C2) and/or third concentration (C3) and/or the combined signal is measured by means of a color reaction or fluorescence detection.

10. The method as claimed in any of the preceding claims, where the protein which can be modified by a vitamin K-dependent γ-carboxylase is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

11. A kit for carrying out the method as claimed in any of the preceding claims, having a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein and having a second antibody (A2)

for immunological determination of a second concentration (C2) of the decarboxylated form of the protein,
characterized in that
first (A1) and second antibodies (A2) is in each case conjugated to a labeling substance, where the labeling substances are selected so that they are able together to generate a combined signal.

12. A kit as claimed in claim 11, where the labeling substance is an enzyme, a fluorescent dye or a quencher.

13. A kit as claimed in claim 11 or 12, where the combined signal is a combined color, a fluorescent signal elicited by the Förster effect or a reduction caused by a quencher in a fluorescent signal.

14. A kit as claimed in any of claims 11 to 13, where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

15. The use of a kit
comprising at least two antibodies selected from a group consisting of a first antibody (A1) for immunological determination of a first concentration (C1) of the carboxylated form of the protein, a second antibody (A2) for immunological determination of a second concentration (C2) of the decarboxylated form of the protein and a third antibody (A3) for immunological determination of a total concentration (C3) of carboxylated and decarboxylated protein,
for carrying out the method as claimed in any of claims 1 to 10.

16. The use as claimed in claim 15, where additionally at least a first competitor (K1) is present for measuring the first concentration (C1), a second competitor (K2) is present for measuring the second concentration (C2) or a third competitor (K3) is present for measuring the third concentration (C3).

17. The use as claimed in claim 15 or 16, where at least one of the antibodies (A1, A2, A3) or competitors (K1, K2, K3) present is conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin.

18. The use as claimed in any of claims 15 to 17, where the first (A1) and the second antibody (A2) are immobilized on a support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane.

19. The use as claimed in claim 18, where the support is a test strip, and the first (A1) and the second antibody (A2) are each absorbed on a separate field of the test strip.

20. The use as claimed in claim 18 or 19, where a third antibody (A3) conjugated to a labeling substance, in particular an enzyme, a fluorescent dye, a quencher, a gold particle, a latex particle, biotin, streptavidin or avidin is present.

21. The use as claimed in any of claims 15 to 20, where the third antibody (A3) is immobilized on the or another support, in particular a plastic, a magnetic particle, a latex particle, a gold particle, a test strip or a membrane.

22. The use as claimed in claim 21, where the support is the or another test strip, and the third antibody (A3) is absorbed on a field of the test strip.

23. The use as claimed in any of claims 15 to 22, where the protein is prothrombin, factor VII, factor IX, factor X, nephrocalcin or osteocalcin.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

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Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: METHOD FOR INDIRECTLY DETERMINING THE BLOOD-CLOTTING STATUS

(54) Bezeichnung: VERFAHREN ZUR INDIREKTEN BESTIMMUNG DES BLUTGERINNUNGSSTATUS

(57) Abstract: The invention relates to a method for indirectly determining the blood-clotting status. The inventive method comprises the following steps: a) collecting body fluids which contain a protein that can be modified by a vitamin K-dependent γ-carboxylase, b) determining at least two concentrations selected from a group consisting of a first concentration C1 of carboxylated protein, a second concentration C2 of decarboxylated protein and an entire concentration C3 of carboxylated and decarboxylated protein, whereby the first concentration C1 is determined using a first antibody A1, the second concentration using a second antibody A2 and the third concentration C3 using a third antibody A3, c) generating a first quotient Q1 from the first C1 and second concentration C2 or generating a second quotient Q2 from the third C3 and first concentration C1 or generating a third quotient Q3 from the third C3 and second concentration C2, whereby a concentration C1, C2, C3 which has not been determined in step b) and which is required for generating the first Q1, the second Q2 or the third quotient Q3 is calculated according to the following relation: C3 C2 = C1 and d) the first, second or third quotient Q1, Q2, Q3 are correlated with the blood-clotting status.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur indirekten Bestimmung des Blutgerinnungsstatus mit folgenden Schritten: a) Entnahme von Körperflüssigkeit, die ein durch eine Vitamin-K abhängige γ-Carboxylase modifizierbares Protein enthält; b) Ermittlung von mindestens zwei Konzentrationen ausgewählt aus einer Gruppe bestehend aus einer ersten Konzentration (C1) an carboxyliertem Protein, einer zweiten Konzentration (C2) an decarboxyliertem Protein und einer Gesamtkonzentration (C3) an carboxyliertem und decarboxyliertem Protein, wobei die erste Konzentration (C1) unter Verwendung eines ersten Antikörpers (A1), die zweite Konzentration unter Verwendung eines zweiten Antikörpers (A2) und die dritte Konzentration (C3) unter Verwendung eines dritten Antikörpers (A3) ermittelt wird; c) Bildung eines ersten Quotienten (Q1) aus erster (C1) und zweiter Konzentration (C2), oder Bildung eines zweiten Quotienten (Q2) aus dritter (C3) und erster Konzentration (C1), oder Bildung eines dritten Quotienten (Q3) aus dritter (C3) und zweiter Konzentration (C2), wobei eine zur Bildung des ersten (Q1), zweiten (Q2) oder dritten Quotienten (Q3) erforderliche und bei Schritt lit. (b) nicht ermittelte Konzentration (C1, C2, C3) gemäß folgender Beziehung: C3 - C2 = C1 errechnet wird; und d) Korrelation des ersten, zweiten oder dritten Quotienten (Q1, Q2, Q3) mit dem Blutgerinnungsstatus.

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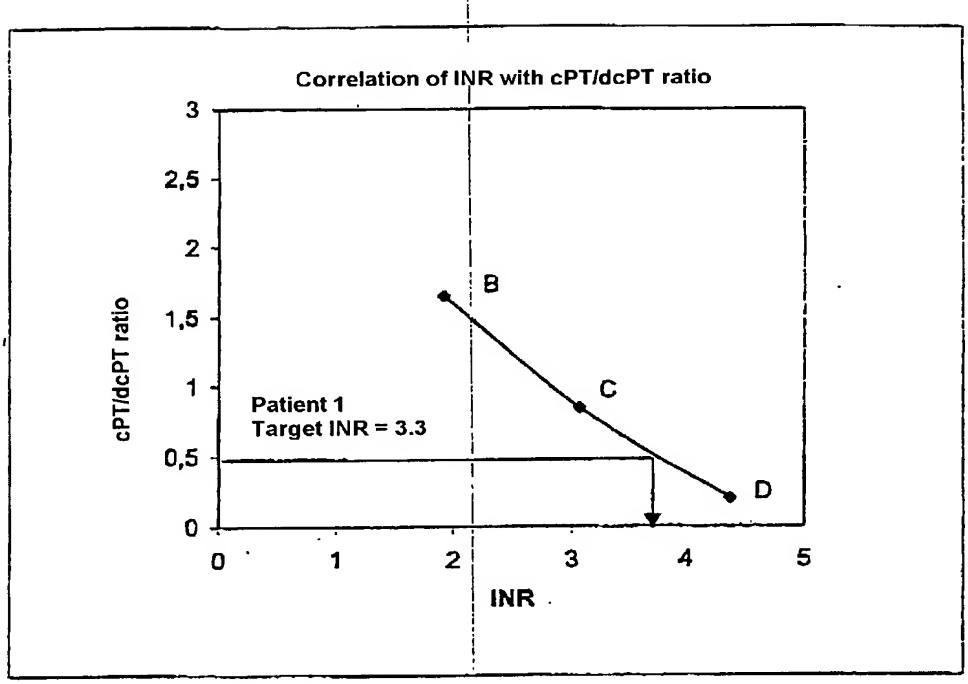
Correlation of INR with cPT/dcPT ratio
cPT/dcPT ratio
3
2,5
2
1,5
1
0,5
0
B
C
D
Patient 1
Target INR = 3.3
0
1
2
3
4
5
INR

Fig. 1

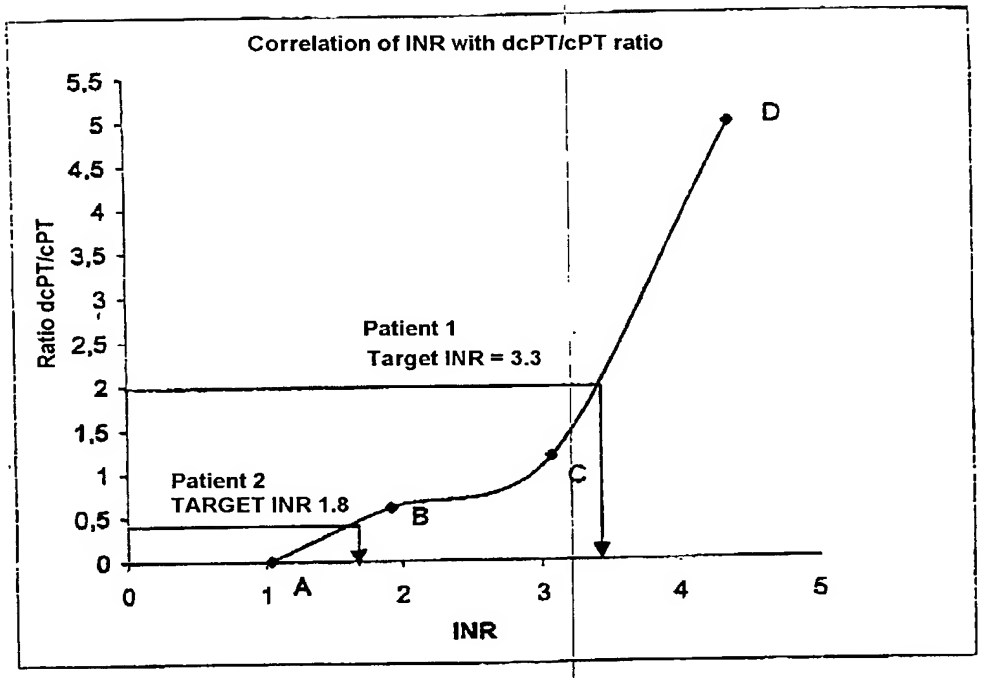
Correlation of INR with dcPT/cPT ratio
Ratio dcPT/cPT
5,5
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Patient 1
Target INR = 3.3
Patient 2
TARGET INR 1.8
A
B
C
D
0
1
2
3
4
5
INR

Fig. 2

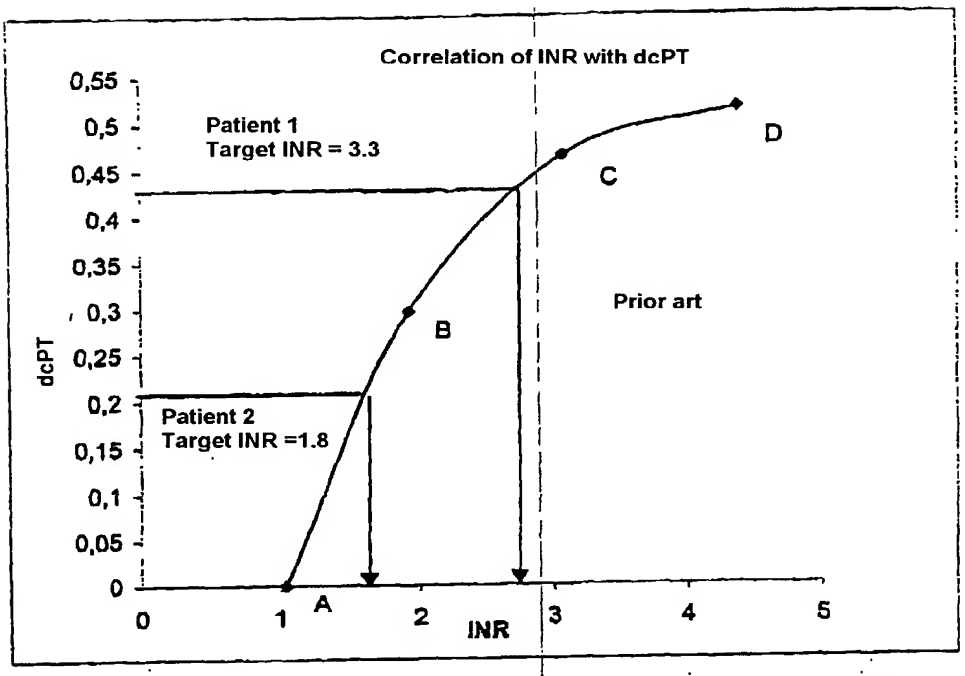
Correlation of INR with dcPT
dcPT
0,55
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Patient 1
Target INR = 3.3
Patient 2
Target INR =1.8
A
B
C
D
Prior art
0
1
2
3
4
5
INR

Fig. 3

Combined Declaration and Power of Attorney

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

100

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COMBINED DECLARATION AND POWER OF ATTORNEY

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As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled *Method For Indirectly Determining the Blood-Clotting Status*, the specification of which:

[] is attached hereto.
[X] was filed on February 14, 2002 as Application Serial No. 10/049,574.
[] was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
WIPO	PCT/DE00/02748	August 11, 2000	[X] Yes [] No
Germany	199 41 447.5	August 31, 1999	[X] Yes [] No
Germany	199 37 654. 9	August 14, 1999	[X] Yes [] No

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